

The $\alpha V\beta 3/\alpha V\beta 5$ integrin inhibitor cilengitide augments tumor response to melphalan isolated limb perfusion in a sarcoma model

Timo L.M. ten Hagen¹, Ann L.B. Seynhaeve¹, Gisela aan de Wiel-Ambagtsheer¹, Ernst A. de Bruijn², Sandra T. van Tiel¹, Curzio Ruegg^{3,4}, Michael Meyring⁵, Matthias Grell⁶, Simon L. Goodman⁶ and Alexander M.M. Eggermont^{1,7}

¹ Department of Surgery, Section Surgical Oncology, Laboratory Experimental Surgical Oncology, Erasmus Medical Center, Rotterdam, The Netherlands

² Department of Experimental Oncology, University of Leuven, Leuven, Belgium

³ Division of Experimental Oncology, Centre Pluridisciplinaire d'Oncologie, University of Lausanne and University Hospital, Lausanne, Switzerland

⁴ Department of Medicine, Faculty of Sciences, University of Fribourg and Swiss Institute for Experimental Cancer Research, NCCR Molecular Oncology, Epalinges, Switzerland

⁵ Merck KGaA, Institute of Drug Metabolism and Pharmacokinetics, Grafting, Germany

⁶ Merck KGaA, Oncology Research and Development, Darmstadt, Germany

⁷ Institut de Cancérologie Gustave Roussy, Villejuif/Paris, France

Isolated limb perfusion (ILP) with melphalan and tumor necrosis factor (TNF)- α is used to treat bulky, locally advanced melanoma and sarcoma. However, TNF toxicity suggests a need for better-tolerated drugs. Cilengitide (EMD 121974), a novel cyclic inhibitor of αV integrins, has both anti-angiogenic and direct anti-tumor effects and is a possible alternative to TNF in ILP. In this study, rats bearing a hind limb soft tissue sarcoma underwent ILP using different combinations of melphalan, TNF and cilengitide in the perfusate. Further groups had intra-peritoneal (i.p.) injections of cilengitide or saline 2 hr before and 3 hr after ILP. A 77% response rate (RR) was seen in animals treated i.p. with cilengitide and perfused with melphalan plus cilengitide. The RR was 85% in animals treated i.p. with cilengitide and ILP using melphalan plus both TNF and cilengitide. Both RRs were significantly greater than those seen with melphalan or cilengitide alone. Histopathology showed that high RRs were accompanied by disruption of tumor vascular endothelium and tumor necrosis. Compared with ILP using melphalan alone, the addition of cilengitide resulted in a three to sevenfold increase in melphalan concentration in tumor but not in muscle in the perfused limb. Supportive *in vitro* studies indicate that cilengitide both inhibits tumor cell attachment and increases endothelial permeability. Since cilengitide has low toxicity, these data suggest the agent is a good alternative to TNF in the ILP setting.

Since the continued growth of solid tumors requires angiogenesis, an anti-angiogenic approach to cancer therapy is logical. Since angiogenesis is mediated in part by adhesion receptors of the integrin family, including the αV (αV) integrins,^{1,2} there is a strong rationale for assessing the effects of integrin inhibitors in cancer treatment. Cilengitide (EMD

Key words: cilengitide, EMD 121974, $\alpha V\beta 3$, isolated limb perfusion, sarcoma, melphalan

Abbreviations: αV : αV ; BSA: bovine serum albumin; CAM: chick chorioallantoic membrane; CR: complete response; c(RGDfV): cyclo(Arg-Gly-Asp-D-Phe-Val); DCE-MRI: dynamic contrast enhanced magnetic resonance imaging; ECM: extracellular matrix; GC-MS: gas chromatography-mass spectrometry; HUVEC: human umbilical vein endothelial cells; IC₅₀: half maximal inhibitory concentration; ILP: isolated limb perfusion; i.p.: intraperitoneal; LC-MS/MS: liquid chromatography with tandem mass spectrometry; LSD: least significant difference; PD: progressive disease; PR: partial response; R²: goodness of fit; RR: response rate; RGD: Arg-Gly-Asp; SD: stable disease; SEM: standard error of the mean; SRB: sulforhodamine-B; TGF- β : transforming growth factor- β ; TNF- α : tumor necrosis factor- α . Additional Supporting Information may be found in the online version of this article.

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Curzio Ruegg's current address is: Pathology, Department of Medicine, University of Fribourg, Fribourg, Switzerland

Correspondence to: T.L.M. ten Hagen, Department of Surgery, Section Surgical Oncology, Erasmus Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, Tel.: +31-10-4087682, Fax: +31-10-4089471, E-mail: t.l.m.tenhagen@erasmusmc.nl

What's new?

The growth of solid tumors requires angiogenesis, a process that is mediated in part by the integrin adhesion receptors $\alpha V\beta 3$ and $\alpha V\beta 5$, which are inhibited by the drug cilengitide. Cilengitide is known to possess anti-tumor and antiangiogenic effects. In this study, it was discovered further to increase tumor response rate in a rat model of soft tissue sarcoma, when administered in conjunction with melphalan. The findings suggest that cilengitide may be a viable alternative to tumor necrosis factor- α in the treatment of locally advanced sarcoma.

121974) is a novel cyclic inhibitor of $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins with both anti-angiogenic and direct anti-tumor effects. We here report a series of experiments investigating its use in combination with melphalan and tumor necrosis factor- α (TNF) in a sarcoma isolated limb perfusion (ILP) model.

$\alpha V\beta 3$ and $\alpha V\beta 5$ integrins mediate the interaction between endothelial cells and components of the extracellular matrix (ECM).^{3,4} Preventing the binding of endothelial cells to ECM ligands results in apoptosis.^{5,6} Direct proof of the importance of αV integrins in tumor growth and endothelial stability comes from studies employing specific antibodies. For example, in a chick chorioallantoic membrane (CAM) assay, the antibody LM609 (which inhibits $\alpha V\beta 3$) reduces tumor growth and invasion,⁷ and integrin antagonists inhibit angiogenesis.^{8,9} The anti- αV -integrin antibody 17E6 inhibits melanoma development in a mouse model¹⁰ and inhibits attachment of endothelial cells to vitronectin and, partially, to fibronectin.

Integrin $\alpha V\beta 3$ binds to Arg-Gly-Asp (RGD) in vitronectin, fibronectin and fibrinogen, among other substrates.^{11–16} RGD cyclic peptides can induce apoptosis in endothelial cells, inhibit angiogenesis and block tumor growth. One such cyclic RGD peptide is cilengitide (EMD 121974), Cyclo-(Arg-Gly-Asp-D-Phe-(N-Me) Val).^{17–19} Cilengitide disrupts VE-cadherin localization at cell junctions, and increases endothelial monolayer permeability²⁰ and disrupts angiogenesis *in vitro*²¹ and *in vivo*.²² In a tumor-induced CAM assay, it inhibits cell-matrix contact and angiogenesis.²³ Co-administration of this anti-integrin improved the efficacy of radioimmunotherapy in breast cancer xenograft²⁴ and glioblastoma orthotopic xenograft model.²⁵ Importantly, apoptosis of both tumor and endothelial cells was significantly increased.^{24,26} Most recently, cilengitide has been shown to reduce the size of breast cancer bone metastases in a nude rat model and increase blood vessel permeability on dynamic contrast enhanced magnetic resonance imaging (DCE-MRI).^{27,28}

ILP with melphalan and TNF is used as salvage therapy in patients with distributed malignant melanoma or soft tissue sarcoma, and a response rate of 76% has been reported in locally advanced soft tissue sarcoma.²⁹ We have previously shown in the ILP system that the alkylating agent melphalan primarily induces tumor cell death while TNF inflicts tumor vascular damage and endothelial cell death.^{30,31} The response rate therefore depends on the direct anti-tumor activity of melphalan combined with the anti-neovascular activity of TNF. A consequence of the latter is increased influx of cyto-

toxic agents such as melphalan and doxorubicin into the tumor, which (probably because of high intra-tumoral pressure) is normally resistant to their access.^{30,32}

Based on the capacity of cilengitide to disrupt active angiogenic endothelium and trigger endothelial-cell apoptosis in neovasculature, we investigated whether its addition to melphalan-based loco-regional treatment of established BN175 soft tissue sarcomas could improve tumor response rate. Our results showed that cilengitide administered shortly before, during and after ILP greatly increased the anti-tumor activity of melphalan. This synergistic effect was not explained by altered pharmacokinetics but was associated with increased levels of melphalan in the tumor compartment. Cilengitide is currently under active investigation as a systemic agent in randomized phase II and phase III clinical trials in tumors including squamous cell cancer of the head and neck and glioblastoma,^{32–35} in which antitumor activity has been reported.³⁶

To complement our *in vivo* ILP studies in sarcoma and explore possible mechanisms of action underlying the phenomena observed, we carried out a series of *in vitro* experiments investigating the effects of cilengitide on BN175 cell adhesion and endothelial permeability. Such effects may account for the increased tumor response rates seen.

Material and Methods

Drugs

Cilengitide (EMD 121974; cyclic(Arg-Gly-Asp-D-Phe-(N-Methyl)-Valine) in physiological saline; 15 $\mu\text{g/ml}$; 25.5 μM) was provided by Merck KGaA, Darmstadt, Germany, and further diluted in saline. Melphalan (50 mg per vial) was diluted in phosphate-buffered saline (PBS) to a concentration of 2 mg/ml. The recombinant human TNF, kindly provided by Dr. G. Adolf (Boehringer Ingelheim GmbH, Vienna, Austria), had a specific activity of 5×10^7 U/mg by the murine L-M cell assay.

Animal tumor model

Male inbred Brown Norway rats (Harlan-CPB, Austerlitz, The Netherlands) were used at a weight of 250 to 300 g and fed a standard diet (Hope Farms, Woerden, The Netherlands) *ad libitum*. Fragments of the syngeneic BN175 soft tissue sarcoma (1 mm³) were implanted subcutaneously in the right hind leg just above the ankle.³⁷ Tumor growth was measured daily by calipers and volume calculated using the

Table 1. Treatment groups and tumor response of soft tissue sarcoma-bearing rats after ILP with cilengitide, TNF and melphalan

Group	Therapy regimen		Tumor response					n
	i.p. Injections ¹	In perfusate ²	PD	SD	PR	CR	RR	
A	Sham	Sham	9	1			0	10
B	Sham	Cilengitide	4				0	4
C	Sham	TNF	9	1	1	1	17%	12
D	Sham	Melphalan	9	2	1		8%	12
E	Sham	Cilengitide + TNF	6				0	6
F	Sham	Cilengitide + melphalan	3				0	3
G	Sham	TNF + melphalan	7	2	1	2	25%	12
H	Sham	Cilengitide + TNF + melphalan	2	1	2	1	50%	6
I	Cilengitide	Sham	7				0	7
J	Cilengitide	Cilengitide	14				0	14
L	Cilengitide	TNF	5				0	5
K	Cilengitide	Melphalan	5		1		17%	6
M	Cilengitide	Cilengitide + TNF ³	5				0	5
N	Cilengitide	Cilengitide + TNF	10			2	16%	12
O	Cilengitide	Cilengitide + melphalan	2	1	2	8	77%	13
P	Cilengitide	TNF + melphalan			3	3	100%	6
Q	Cilengitide	Cilengitide + TNF + melphalan	2	0	3	8	85%	13
R	Cilengitide before ILP	Cilengitide + TNF + melphalan	3 ⁴	1	1		20%	5
S	Cilengitide after ILP	TNF + melphalan	3		1	1	40%	5

Animals were treated as depicted in Figure 1.

¹Rats were injected i.p. 2 hr before and 3 hr after ILP.

²ILP was started with sham or cilengitide and after 10 min melphalan or TNF was added.

³TNF was added with cilengitide at the beginning of the 30-min perfusion, in contrast to other groups, in which TNF and/or melphalan was present for only 20 min.

⁴Although an initial response was observed, rapid regrowth occurred showing PD at day 10.

Abbreviations: ILP, isolated limb perfusion; TNF- α , tumor necrosis factor- α ; i.p., intraperitoneal; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response; RR, response rate (PR plus CR).

formula $0.4 \times (A^2 \times B)$ where B represents the largest diameter and A the diameter perpendicular to it. Rats were sacrificed when tumor diameter exceeded 25 mm or at the end of the experiment (4 weeks). Their temperature was monitored daily using a transponder implanted intraperitoneally (i.p.; ELAMS, Electronic Laboratory Animal Monitoring System, BioMedic Data Systems, Inc., Seaford, DE, USA). All studies were conducted in accordance with protocols approved by the Committee on Animal Research of the Erasmus Medical Center.

Treatment protocol

Tumors were allowed to grow to a diameter of 12 to 14 mm before rats were randomly assigned to the treatment groups listed in Table 1. These groups were designed to investigate the effects of perfusing various combinations of melphalan, TNF and cilengitide with or without the additional i.p. administration of cilengitide before and after the ILP procedure itself. The i.p. administration pre- and post-ILP was intended to optimally saturate available $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins. Saline was used as a control in both the i.p. and perfusion settings.

Figure 1 shows the drug administration schedules for the different groups. During ILP, cilengitide (500 μ g, 170 μ M), melphalan (40 μ g, 8 μ g/ml) and/or TNF (50 μ g, 10 μ g/ml) were added to the circuit containing a total volume of 5 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands).

Rats were injected i.p. with 50 mg/kg cilengitide or saline 2 hr before and 3 hr after ILP. Figure 1 also shows the times at which perfusate was sampled for determination of melphalan level, the point at which tissues were taken and the times before and after ILP when serum was sampled.

Isolated limb perfusion

Animals were anesthetized with Hypnorm and ketamine (Janssen Pharmaceutica, Tilburg, The Netherlands). The femoral vessels were approached through an incision parallel to the inguinal ligament after systemic heparin administration of 50 IU (Leo Pharmaceutical Products, Weesp, The Netherlands) to prevent coagulation in the collateral circulation and in the perfusion circuit. The femoral artery and vein were cannulated with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 mm inner diameter, 1.19 mm outer

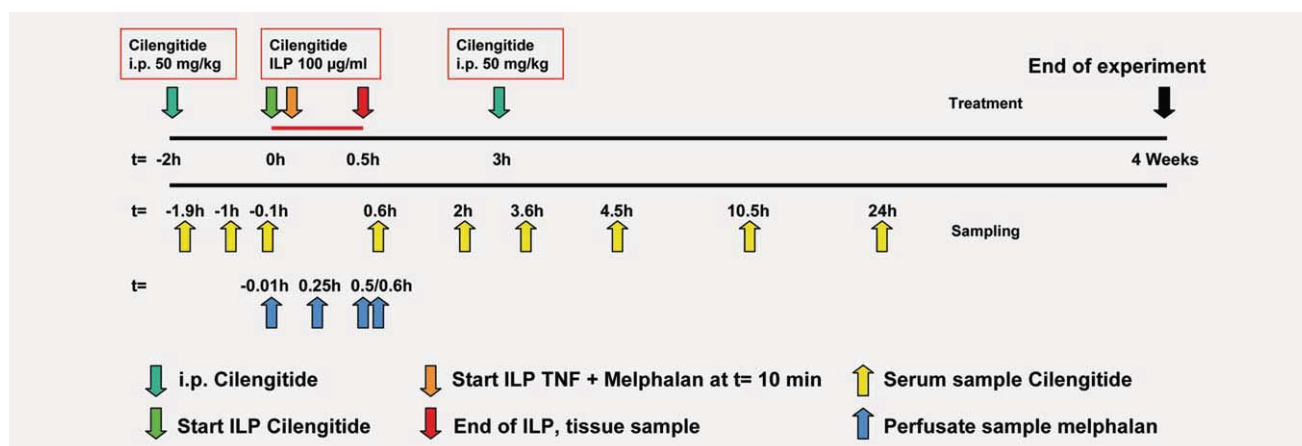


Figure 1. Time-line of treatment and sample collection. ILP, isolated limb perfusion; i.p., intraperitoneal; TNF- α , tumor necrosis factor- α .

diameter, respectively; Dow Corning, Ann Arbor, MI, USA). A tourniquet around the groin temporarily occluded collaterals. An oxygenation reservoir filled with 5 ml Haemacel and a low-flow roller pump (Watson Marlow type 505 U, Falmouth, UK) were included in the circuit.

Cilengitide, melphalan and TNF were added as a bolus to the circuit reservoir. Perfusate was circulated at 2.4 ml/min. Our previous studies involved perfusion with TNF and melphalan for 30 min. However, in these experiments ILP with melphalan and/or TNF lasted 20 min since the first 10 min were used to administer cilengitide or saline. A washout with 5 ml oxygenated Haemacel was performed at the end of perfusion. During ILP and washout, the hind leg was kept at a constant 38 to 39°C by a warm-water mattress around the leg. Limb function was subsequently monitored daily to assess local toxicity.

Tumor response

Response was classified as progressive disease (PD) if volume increased by more than 25%; stable disease (SD) if tumor remained within $\pm 25\%$ of the volume during perfusion; partial response (PR) if volume decreased by 25 to 90%; and complete response if volume decreased to less than 10% of the initial value. The tumor response ratio was calculated by dividing the tumor volume at day 8 by the volume at day 0. Tumor response after combined therapy that was greater than the sum of the responses obtained with the individual component drugs was taken as evidence of synergy.³⁸

Histopathology

Directly after ILP, tumors were excised, stored in formalin, and embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin and eosin using standard procedures. Three or four tumors in each experimental group were evaluated blind and at least six slides from each tumor examined (Leica DM-RXA light microscope) and photographed (Sony 3CCD DXC 950 camera). Tumor tissues were also stained for the presence of endothelial cells.

Acetone-fixed frozen sections were post-fixed in 4% paraformaldehyde for 30 min and incubated for 1 hr with mouse-anti-rat CD31PE (Becton Dickinson, Breda, The Netherlands) diluted 1:50 in PBS with 5% rat serum.

Pharmacokinetics

Serum and perfusate samples were taken from at least three rats per group at the times shown in Figure 1. Directly after washout, tumor and part of the muscle on the infused and non-infused hind limbs were excised. Samples were immediately frozen in liquid nitrogen and stored at -80°C . Tissues were homogenized in 2 ml acetonitrile (Pro 200 homogenizer, Pro Scientific, Oxford, CT) and centrifuged at 2,500 g. Melphalan was measured in these supernatants and in serum and perfusate samples by gas chromatography-mass spectrometry (GC-MS). *p*-(Bis(2-chloroethyl)amino)-phenylacetic methyl ester was used as internal standard. Samples were extracted over tri-functional C18 silica columns. After elution with methanol and evaporation, the compounds were derivatized with trifluoroacetic anhydride and diazomethane in ether. The stable derivatives were separated on a methylphenylsiloxane GC capillary-column and measured selectively by single-ion monitoring GC-MS in the positive EI mode.³⁹

Cilengitide in serum was measured using liquid chromatography with tandem mass spectrometry (LC-MS/MS) and the cyclic peptide cyclo(Arg-Gly-Asp-D-Phe-Val) (c(RGDfV)) as an internal standard. Stock solutions of c(RGDfV) were prepared in acetonitrile/methanol/water (1:1:1, v/v/v) and further diluted with methanol/water (2:98, v/v). Aliquots were mixed with internal standard solution (1:80, v/v), extracted over an Oasis HLB column (50 \times 1 mm; Waters, Saint-Quentin, France), and resolved on a reversed-phase column (LiChroCart 30-2 Purospher, RP8e; Merck KGaA, Darmstadt, Germany) on a gradient from 100% (0.1%) formic acid to 10% (0.1%) formic acid/90% acetonitrile (v/v). Detection was carried out by multiple reaction monitoring in the positive ion mode using an API-3000 mass spectrometer (AB Sciex, Darmstadt, Germany). Mass transitions of $M+H^+$

m/z 589 to 312 and *m/z* 575 to 312 (precursor and product ions) were used for cilengitide and internal standard c(RGDfV), respectively.

Statistical analysis

Response rates were subjected to analysis-of-variance and post hoc to the least significant difference (LSD) multiple comparison test. Tumor volume was evaluated by repeated measures analysis of variance on days 4, 8 and 12. All statistical tests were two-sided; $p < 0.05$ was considered significant. The possible synergy of combined treatment was assessed as described previously.³⁸ Data were analyzed by the Mann-Whitney *U* test (exact significance [$2 \times$ (one-tailed significance)]) using SPSS version 10.0 for Windows 2000. Calculations were performed using GraphPad Prism v3.0 and SPSS v11.0 for Windows 2000.

Associated in vitro studies

Cell attachment and attachment inhibition assays were conducted to confirm that the target integrins are expressed by BN175 sarcoma cells and to investigate the inhibition of cell adhesion by cilengitide. BN175 cells in log-phase growth were harvested, washed and re-suspended in culture medium (RPMI-1640 supplemented with 0.5% bovine serum albumin [BSA]). In culture medium, 2.5×10^4 cells per well were allowed to adhere for 1 hr at 37°C onto 96-well plates pre-coated with the ECM molecules human plasma fibronectin, vitronectin or laminin I (Sigma-Aldrich, Zwijndrecht, The Netherlands) before washing free of non-attached cells and quantification by assay for lysosomal hexosaminidase against an external standard curve.⁴⁰ To assay for adhesion inhibition, 96-well plates were coated with vitronectin (2 mg/ml) and 2.5×10^4 cells per well allowed to adhere for 1 hr in the presence of serially diluted cilengitide before washing and quantification of attached cells as above.

The *in vitro* response of endothelial and tumor cells to cilengitide was also investigated. BN175 cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (Cambrex Bio Science, Verviers, Belgium). Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion.⁴¹ Each isolate was derived from an individual umbilical vein and used for experiments at passage 5 or 6. HUVEC were cultured in medium containing human endothelial-SFM (Invitrogen, Breda, The Netherlands), 20% heat-inactivated newborn calf serum, 10% heat-inactivated human serum (Cambrex Bio Science, Verviers, Belgium), 20 ng/ml human recombinant basic fibroblast growth factor (Peprotech EC Ltd, London, UK), 100 ng/ml human recombinant epidermal growth factor (Peprotech EC Ltd). HUVEC were cultured on plates coated with vitronectin (1 µg/ml; Promega, Leiden, The Netherlands), fibronectin (10 µg/ml; Roche Diagnostics, Almere, The Netherlands) or gelatin (10 mg/ml; Sigma, Zwijndrecht, The Netherlands).

BN175 cells and HUVEC were added in 100-µl aliquots to 96-well plates at 6×10^3 cells per well and allowed to

grow for 24 hr. Cells were incubated at 37°C in 5% CO₂ for 72 hr in the continuous presence of various concentrations of cilengitide and melphalan in a total volume of 100 µl. Cell growth was measured using the Sulforhodamine-B (SRB).⁴² The optical density was read at 540 nm. Cell growth was calculated using the formula: cell growth = (OD test well/OD control) \times 100 percent. The drug concentration reducing absorbance to 50% of the control (IC₅₀) was determined from the growth curves. The experiments were repeated at least five times.

To evaluate transendothelial monolayer-permeability, HUVEC were plated in a transwell (Corning BV, Schiphol, The Netherlands; 0.4 µm pore size, 6.5 mm diameter, polyester filters), coated with vitronectin (1 µg/ml) or fibronectin (10 µg/ml), at a density of 1.2×10^4 cells per well. In the lower compartment, 1 ml of HUVEC medium was added. Two days after seeding, a confluent monolayer had formed. Non-adhering cells were removed and the medium replaced with 250 µl of 50, 5, 0.5 or 0 µg/ml cilengitide together with 50 µl fluorescein (0.5 mg/ml). At 0.25, 0.5, 1, 2, 4, 8 and 24 hr, 50 µl medium of the lower chamber was taken and fluorescence activity was measured at excitation 490 nm and emission 530 nm, in a Victor² 1410 multilabel counter (Wallac, Turku, Finland). Induction of permeability was indicated by a higher concentration of fluorescein in the lower chamber of the transwell, relative to untreated controls.

Results

Tumor response and volume

No responses were observed in sham-treated animals or when cilengitide was administered alone (either in the perfusate, as an i.p. injection or both) (Table 1, groups A, B, I and J). ILP with melphalan alone had a minor effect (8% response rate [RR] in 12 animals in group D). The likelihood of response was modestly improved when i.p. cilengitide was administered before and after melphalan perfusion (RR 17%, group L).

In the absence of i.p. injection, the addition of cilengitide to melphalan ILP did not lead to any responses (group F). However, when ILP with melphalan plus cilengitide was preceded and followed by i.p. cilengitide (group O), the combination produced a RR of 77%. Eight of the 13 animals treated with this regimen had a complete response (CR). The RR in this group was highly significantly greater than the RR in animals treated with ILP using melphalan alone (17% RR, $p = 0.002$) or cilengitide alone (when the RR was 0). The fact that this RR so greatly exceeded that seen in groups treated with cilengitide or melphalan alone provided evidence of synergy, using the definition described above.

The effect of administering i.p. cilengitide in conjunction with ILP using cilengitide and TNF (rather than melphalan) was considerably less, although two CRs were observed among 12 animals treated (16% RR in group N). The fact that this combination was only minimally effective may be

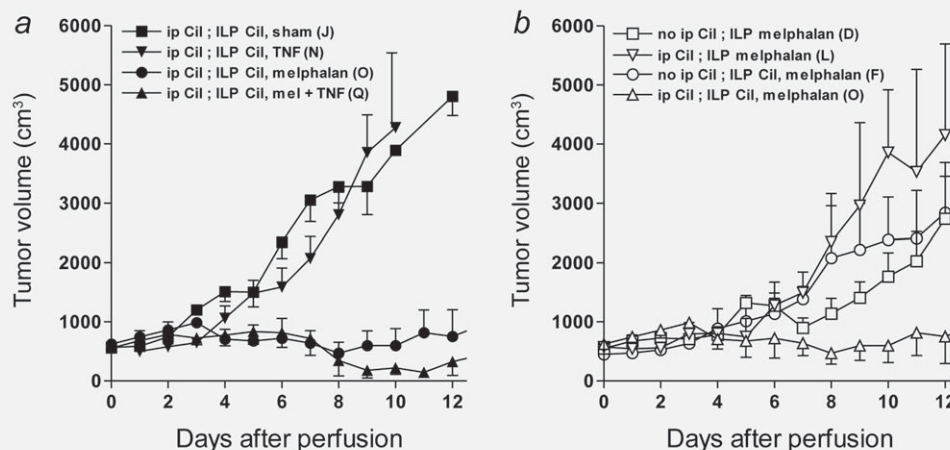


Figure 2. Tumor volume (mean \pm SEM) with time in rats treated with cilengitide in combination with melphalan-based isolated limb perfusion. (a) All rats received cilengitide (Cil) as an intraperitoneal (i.p.) injection and as bolus in the perfusate. Figures in brackets refer to the groups listed in Table 1. (b) Different regimens of cilengitide with melphalan were used. The number of animals per group is shown in Table 1. ILP, isolated limb perfusion; TNF- α , tumor necrosis factor- α ; mel, melphalan; SEM, standard error of the mean.

accounted for by the fact that cilengitide and TNF have the same target, namely tumor endothelial cells.

The activity of ILP with melphalan plus TNF in the absence of any cilengitide was evident in a RR of 25% (group G). Interestingly, a previous study found that perfusion with melphalan plus TNF for the full 30 min resulted in a RR of around 75% in this model. The shorter duration of exposure in the present series of experiments may therefore have substantially reduced activity. However, giving cilengitide pre- and post-ILP with melphalan plus TNF resulted in a 100% RR including three CRs in six animals treated (group P). Among animals receiving i.p. cilengitide before and after ILP with cilengitide added to melphalan and TNF, the RR was 85%, again including eight CRs (group Q). The RR was considerably lower (20%) when i.p. cilengitide following the ILP was omitted (group R). Comparison of the 40% RR in group S with the 100% RR in group P suggests that omitting the pre-ILP administration of cilengitide also appreciably reduces the likelihood of response.

Figure 2 shows change in tumor volume over the 12 days following treatment with cilengitide in combination with ILP. Volumes were measured daily. In panel A, all groups received cilengitide as an i.p. injection before and after ILP and also had cilengitide added as a bolus to perfusate.

Tumor volumes increased steadily over this period in animals that were treated with cilengitide but did not receive melphalan or TNF in their ILP (group J in Table 1). A very similar rate of increase was seen in animals whose ILP contained both cilengitide and TNF (group N). However, ILP with cilengitide plus melphalan preceded and followed by i.p. cilengitide suppressed tumor growth for the period of study (group O); and median tumor volume showed a small decrease in animals perfused with cilengitide plus both melphalan and TNF (group Q).

Panel B shows tumor volumes for four groups treated with various combinations of cilengitide and melphalan. Treatment with cilengitide i.p. pre- and post-ILP using melphalan alone did not prevent tumor growth (group L). An intermediate degree of growth inhibition was achieved in animals treated with melphalan plus cilengitide ILP even in the absence of i.p. drug (group F). However, only three animals (all of whom had PD) were studied. Perhaps surprisingly, group D, which had no i.p. cilengitide and ILP with melphalan alone, showed some inhibition of tumor growth despite an RR of only 8%.

Although many animals received high doses of cilengitide both locally and systemically, no toxicities attributable to the agent were evident through observation of the animals or assessment of their weight and limb function (data not shown). A maximum weight loss of 10% was observed with no difference between the treated groups, and weight loss recovered in most animals. In addition, no abnormal behavior (motility, hair, lethargy, feces) was observed when cilengitide was given systemically and/or through ILP. In all rats treated with cilengitide, alone or in combination with melphalan, limb function remained normal and no edema was observed.

Histopathology

Histopathology showed no effect on tumor tissue or vasculature in sham-treated animals and in those administered ILP containing either melphalan or TNF alone (Fig. 3a, left-hand column). However, the co-administration of cilengitide and either melphalan or TNF dramatically affected the endothelial integrity of tumor blood vessels (Figs. 3a and 3b). Hemorrhage was observed in all animals treated with cilengitide combined with melphalan, TNF or both. Immunohistology revealed clear disruption of the endothelial layers. Large areas

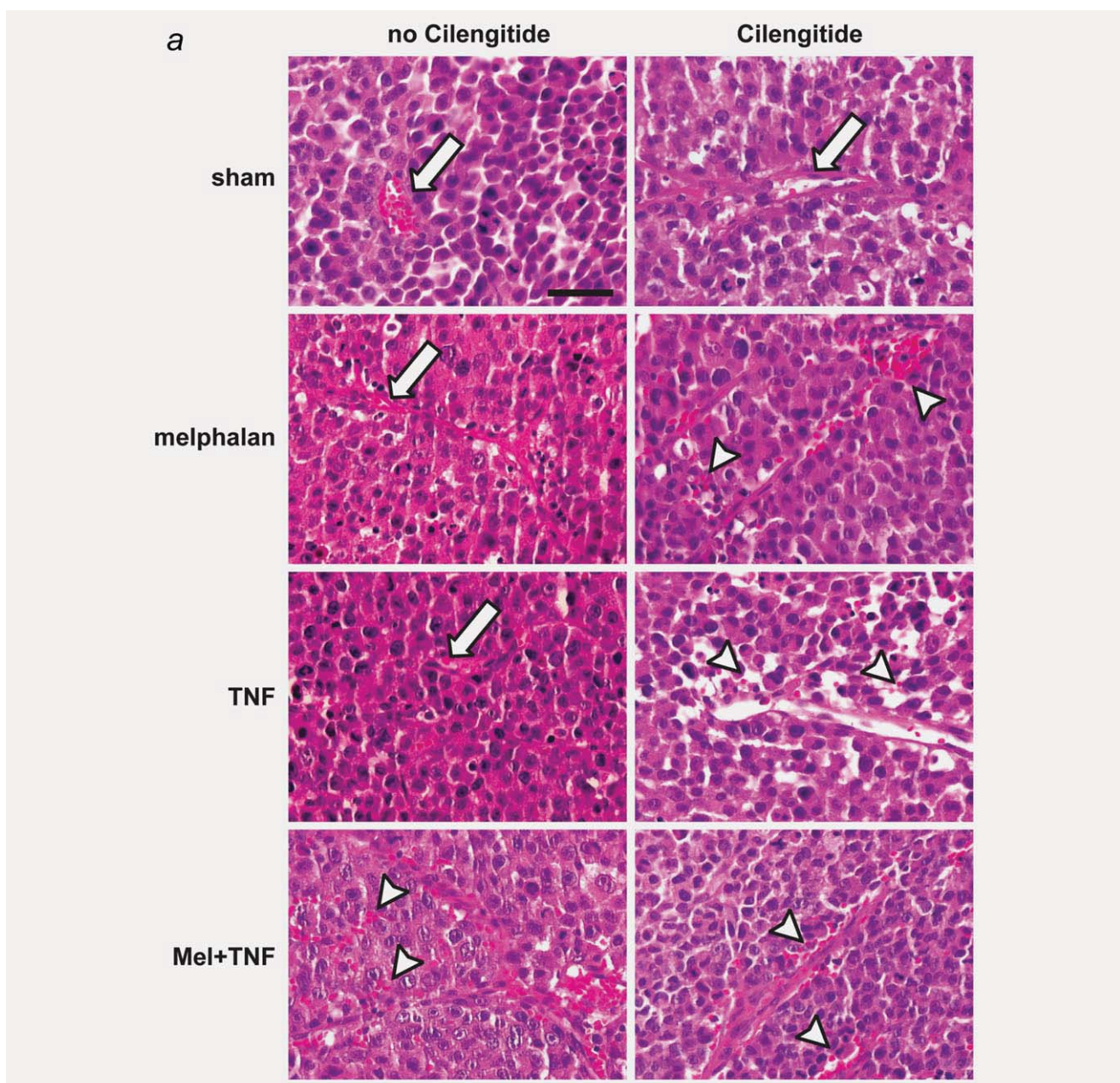


Figure 3. Histopathology of soft tissue sarcomas after cilengitide treatment in combination with a melphalan-based ILP. (a) After treatment, tumors were excised from two rats per group. Hematoxylin and eosin staining showed no effect on tumor or tumor-related vasculature (left panel, open arrows) when animals were treated with sham, melphalan or TNF alone. Confirming previous results, ILP with melphalan plus TNF resulted in marked vascular damage (arrow heads) accompanied by hemorrhage and edema (asterisks). Administration of cilengitide (right panel) alone had no noticeable effect on tumor histology or associated vasculature (open arrow). However, when cilengitide was combined with melphalan, TNF or melphalan plus TNF massive vascular destruction (open arrows heads) and hemorrhage (asterisks in Supporting Information Fig. 3) were observed. Original magnification $\times 40$ (bar 50 μm). (b) Damage to tumor-associated vasculature visualized by CD31 staining. Perfusion with cilengitide alone had marginal effect on tumor vessel integrity or diameter (closed arrows), while treatment with a combination of cilengitide and melphalan, TNF or melphalan plus TNF resulted in profound loss of vascular integrity (open arrows) and widening of the vascular lumen (arrow head). Original magnification $\times 16$ (bar 100 μm). TNF- α , tumor necrosis factor- α ; mel, melphalan.

of tumor necrosis were observed when cilengitide was combined with melphalan, TNF and melphalan plus TNF (Supporting Information Fig. 3). These results were confirmed by TUNEL staining, showing increased numbers of TUNEL positive cells both stained for CD31 and tumor cells, when cil-

engitide in combination with melphalan was used (data not shown). However, although ILP with TNF plus cilengitide resulted in abundant tumor necrosis, this was not translated into tumor response (groups E, M and N), suggesting that a cytotoxic agent is required to achieve tumor regression.

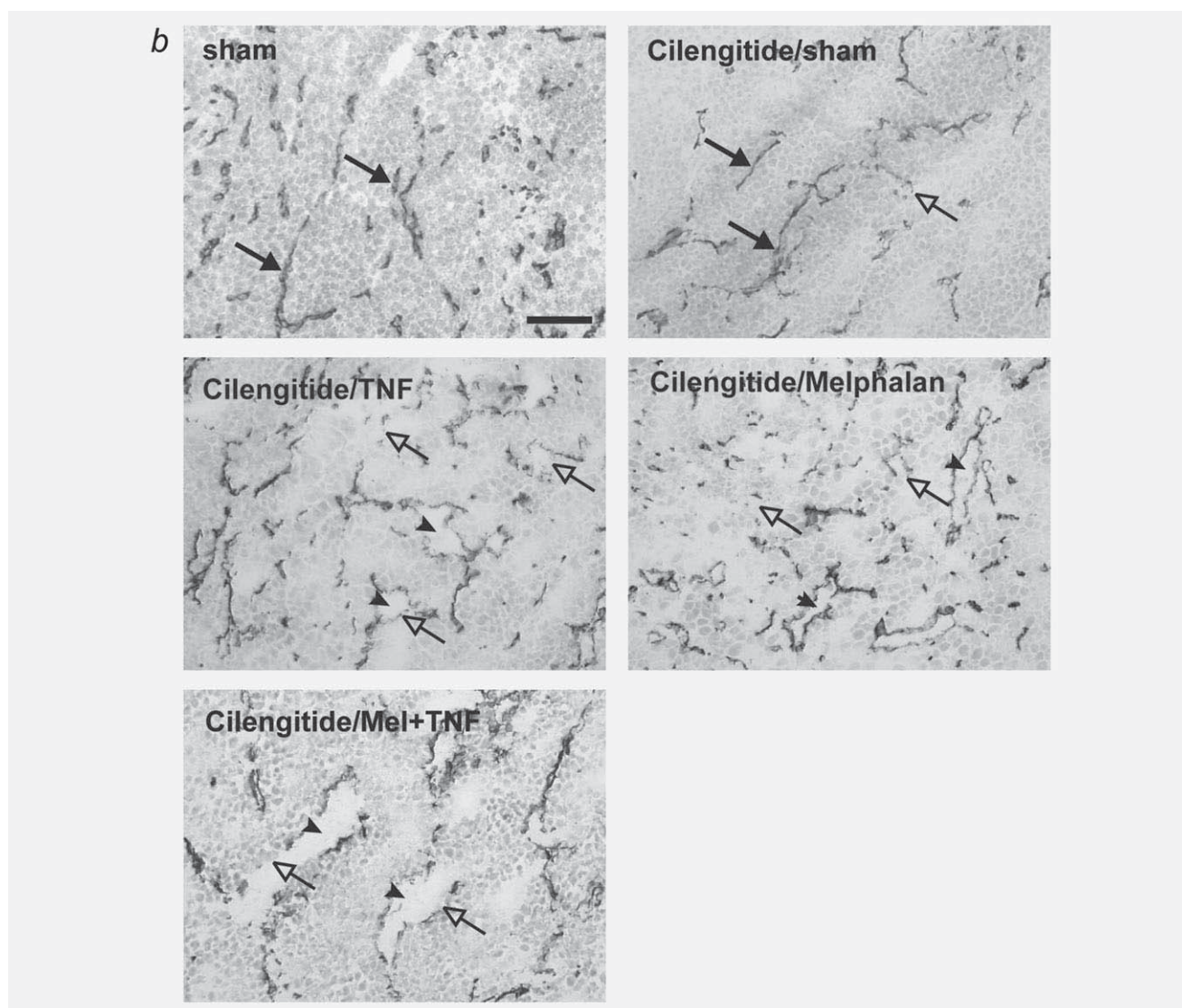


Figure 3. Continued

The inflammatory response in tumor tissue after ILP was studied by analyzing the accumulation and distribution of leukocytes. Only the number of granulocytes was significantly affected by the treatment. Only in the cilengitide alone treated group (group J) granulocytes increased significantly at 0 hr and 12 hr after ILP compared with no cilengitide. At 24 hr and 48 hr granulocyte numbers decreased rapidly, which recovered at 72 hr (data not shown).

Pharmacokinetics

To investigate the mechanisms underlying the improved response resulting from the addition of cilengitide to melphalan-based ILP, intratumor levels of melphalan were measured. ILP with melphalan alone resulted in a mean 0.6 μg melphalan per gram tumor tissue (Fig. 4). Depending on the dosing schedule, this rose to between 1.9 and 4.2 $\mu\text{g/g}$ tumor tissue with the addition of cilengitide. This represents a three to sevenfold increase relative to group D animals treated with

melphalan ILP alone ($p < 0.05$). Melphalan level in tumor was significantly increased both by cilengitide added to the perfusate and by its administration i.p. The effect of adding a bolus of cilengitide to the perfusate was comparable to that of co-perfusing melphalan and TNF.

The addition of cilengitide did not significantly increase the concentration of melphalan in healthy muscle from the ILP-perfused right hind limb. Drug levels in the non-perfused left hind limb were minimal.

In the rat model studied, the systemic pharmacokinetics of i.p. cilengitide were not affected by ILP with cilengitide alone or ILP with cilengitide plus melphalan, TNF or both (Fig. 5). Systemic cilengitide levels reached around 20 $\mu\text{g/ml}$ (approximately 35 μM) within 10 min of i.p. administration and continued to rise to approximately 40 $\mu\text{g/ml}$ (approximately 70 μM) in the first hour. Thereafter cilengitide levels in serum dropped with an elimination half-life of 2.1 hr. The administration of cilengitide during ILP did not affect

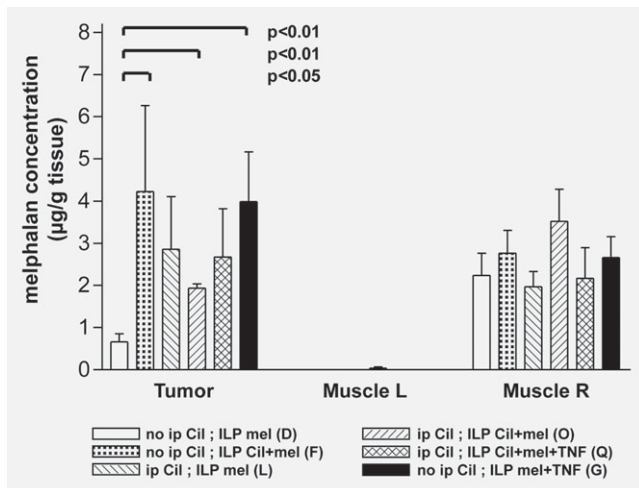


Figure 4. Accumulation of melphalan in tissue after treatment with cilengitide and melphalan-based isolated limb perfusion. Values are shown for tumor, muscle from the non-perfused (left hind limb) and perfused (right) limb. Data represent mean \pm SEM of six independent rats measured in duplicate. Both the addition of cilengitide to the perfusate and its intraperitoneal (i.p.) injection increased accumulation of melphalan in the tumor but not in healthy tissue. Cil, cilengitide; ILP, isolated limb perfusion; mel, melphalan; TNF- α , tumor necrosis factor- α ; SEM, standard error of the mean.

systemic levels due to the relatively low concentration used and the thorough washout at the end of the perfusion. Data were fitted with a one-phase exponential decay with a goodness of fit (R^2) of 0.93.

Supportive in vitro studies

The tumor cells used in the present study were shown to express the integrins targeted by cilengitide. *In vitro* studies demonstrated that BN175 rat sarcoma cells attached to surfaces coated with the ECM molecules vitronectin, fibronectin and laminin. This attachment to vitronectin was inhibited by cilengitide in a dose-dependent manner with an IC_{50} of 4 μ M (SLG: unpublished data).

The direct effect of cilengitide on BN175 tumor cells and HUVECs was also investigated. BN175 cells were exposed to cilengitide alone or combined with melphalan or TNF for 72 hr. A dose-response effect on tumor cells was observed for cilengitide. The addition of melphalan to cilengitide increased cell death of both BN175 cells and HUVECs. However, the addition of TNF had no effect (data not shown). The dose-dependent growth inhibition of HUVECs by cilengitide varied according to the matrix on which cells were cultured, endothelial cells on vitronectin seeming slightly more sensitive than cells on fibronectin (Supporting Information Fig. 2a).

In the sarcoma model used, higher tumor response was associated with an augmented accumulation of melphalan in tumor tissue. Disruption of the vascular lining was evident on histology, suggesting that higher melphalan concentrations

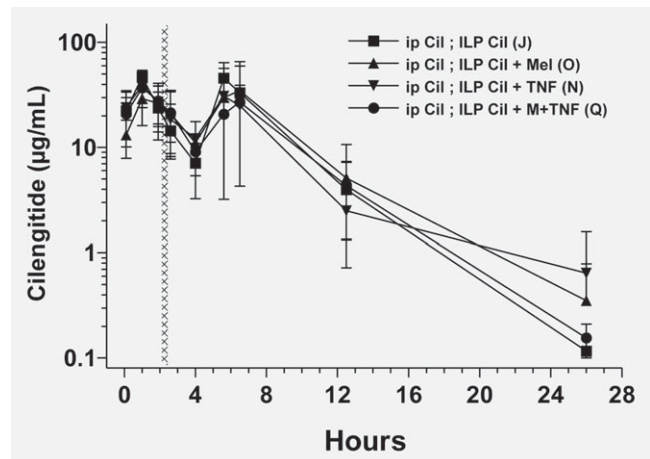


Figure 5. Pharmacokinetics of cilengitide in serum of rats during treatment as depicted in Figure 1. Bars show mean \pm SD of three independent rats. Cilengitide was injected 2 hr before and 3 hr after the period of isolated limb perfusion (hexed region). Serum concentrations increased sharply after intraperitoneal (i.p.) injection followed by a single-phase clearance with a half-life of 2.1 hr. Cil, cilengitide; ILP, isolated limb perfusion; Mel, melphalan; TNF- α , tumor necrosis factor- α ; SD, standard deviation.

resulted from improved intratumoral drug delivery. When HUVEC monolayers were exposed to 50 μ g/ml cilengitide, which is below the maximum theoretical level reached during the ILP (100 μ g/ml), endothelial permeability increased 3.5-fold within 30 min, and continued to increase to a maximum of fivefold in 1 hr (Supporting Information Fig. 2b). At a cilengitide concentration of 0.5 μ g/ml, endothelial cell morphology had changed so that they no longer adhered on vitronectin (data not shown).

Discussion

αV integrins are expressed by the active endothelial cells of the tumor neovasculature, and expression is comparatively low on quiescent endothelia.^{3,7-9,43} Agents directed at these integrins should therefore have a degree of tumor specificity in their effects. Cilengitide inhibits cell-matrix contact and angiogenesis in a tumor-induced CAM assay²³ and increases apoptosis of activated endothelial cells in tumor neovasculature and of tumor cells themselves in animal models.^{24,26} Exploratory *in vivo* studies demonstrated that cilengitide disrupts cell adhesion mediated by $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins.

We investigated whether addition of cilengitide to melphalan-based loco-regional treatment of established soft tissue sarcomas could improve tumor response. A cilengitide-melphalan combination resulted in a 77% response rate while cilengitide or melphalan alone resulted in RRs of 0 and 8%, respectively. Our results show that it is crucial to pre-load animals with cilengitide, which we suggest saturates available integrins and pre-sensitizes target cells to melphalan. Tumor histology showed that the addition of cilengitide to melphalan resulted in loss of vascular endothelial integrity while leaving normal vessels intact, leading to massive

intra-tumoral hemorrhage. Tumor vasculature appeared to have few endothelial cells, consistent with cilengitide-induced cell detachment.

As has been seen previously with TNF, the addition of cilengitide during ILP augmented the accumulation of melphalan in the tumor.^{30,32} Our data are consistent with previous observations suggesting that the effect of adding TNF to melphalan-based ILP is due principally to increased accumulation of melphalan in tumor tissue.²² They further suggest that the same mechanism underlies the enhanced response rates achieved by the addition of cilengitide to ILP.

This could result from improved perfusion of the tumor or from increased leakiness of the tumor vasculature. The latter explanation is supported by the hemorrhaging we observed and the reported increased permeability of an endothelial monolayer *in vitro*.²⁰ Recently, cilengitide has been reported to increase vessel permeability during metastatic bone colonization in a rat breast cancer model.²⁷ The wider therapeutic importance of any cilengitide-induced increase in vascular permeability during ILP has still to be demonstrated, but it is plausible to suggest our results could have implications for the treatment of, for example, melanoma.

The exploratory *in vitro* experiments indicate that a direct effect of cilengitide on tumor cells may also play a role since cell kill was observed at a relatively low concentration. α V integrins are involved in processes including attachment, migration, regulation of matrix metalloproteinase and trans-

forming growth factor (TGF)- β activation, angiogenesis and apoptosis.^{8,9,44–48} It is therefore plausible that inhibiting α V-integrin-ligand interaction should reduce tumor progression. As a single agent, cilengitide inhibits growth of brain tumors²³ and has modest activity in recurrent clinical glioblastoma,³⁶ an activity which appears to be amplified by co-therapy with ionizing radiation.^{25,49} In the present study, as in others such as that of Mikkelsen *et al.*,²⁵ cilengitide alone did not reduce tumor growth but it is possible that this was because our model involved only a single short exposure to the drug.

Our data support the view that cilengitide may approach or match the biologic activity of TNF in ILP. The advantage of cilengitide over TNF is its lack of toxicity. In patients, cilengitide has been reported to be well tolerated up to a dose of 2,400 mg/m²/infusion.⁵⁰ If replicated in other therapeutic contexts, the strong cytotoxic-sensitizing effect of cilengitide, combined with its tolerability, suggests a potential not only in ILP but also in the wider context of systemic combination chemotherapy.

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